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Supplemental Data

Synthetic Lethal Screening Identifies Compounds Activating Iron-Dependent, Nonapoptotic Cell Death in Oncogenic-RAS-Harboring Cancer Cells

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Supplemental Experimental Procedures

Primary Screening

Daughter plates were prepared by diluting mother plates 75-fold in DMEM to obtain a compound concentration of 53.3 μg/mL with 1.33% DMSO in 384-deep-well polypropylene plates (Greiner, cat.#781270). Assay plates were prepared by seeding 1,000 BJ-TERT/LT/ST/RAS^{V12} cells per well in 36 μL of growth media in black, clear-bottom, 384-well plates (Corning Inc., cat. #3712). Columns 3-22 and rows C through N were treated with compounds from a daughter library plate by transferring 4 μL from the daughter library plate. The final compound concentration in assay plates was 5 μg/mL with 0.1% DMSO. All liquid handling was carried out using a Biomek FX AP384 module (Beckman Coulter). The assay plates were incubated for 48 h at 37°C in a humidified incubator containing 5% CO_2. Cell viability was measured using alamar blue (Invitrogen, cat.#DAL1100); subsequently, percent growth inhibition was calculated. All experiments were performed in triplicate and median percent growth inhibition value was used for analyzing data.

Retesting Compounds in Dilution Series in Four BJ Cell Lines

Empty “step-daughter plates” were filled with 50 μL DMEM, except for columns 3 and 13, where 100 μL of hit compound solution were cherry-picked from daughter plates. After compound transfer, 2-fold dilution series across columns 3 through 12 and columns 13 through 22 was made by transferring 50 μL of compound solution to the next column successively (10-point dilution series), with extensive mixing. Hitpicking and 2-fold dilution series were carried out using a Biomek FX Span-8 module (Beckman Coulter). Assay plates were prepared by seeding 1,000 BJ-TERT, BJ-TERT/LT/ST, and BJ-TERT/LT/ST/RAS^{V12} and DRD cells per well in 36 μL growth medium. Cells in assay plates were treated with compounds from step-daughter plates by transferring 4 μL. The final concentration of compound was 5.33 μg/mL to 0.01 μg/mL in this 10-point, 2-fold dilution series. Assay plates were returned to the culture incubator and maintained for 48 h before adding alamar blue.

Counter Screening with Bioactive Compounds

Bioactive compounds listed in Table S1 were dissolved in growth medium at 10x final concentration and aliquoted into microcentrifuge tubes. 100 μL of each 10x solution was transferred to column 1 to column 12 or column 13 to column 24 of a single row in 384-well daughter plate. We named this plate ‘10x bioactive plate’. In order to prepare lethal compound solutions, we dissolved RSL5, RSL3 and erastin in growth medium at a concentration of 400 μg/mL, 100 μg/mL, and 100 μg/mL, respectively. Empty daughter plates were filled with 50 μL growth media except for column 5 and 13, where 100 μL of each lethal solution was transferred. After lethal solution transfer, 2-fold dilution series across column 5 to 12 and column 13 to 20 was made by transferring 50 μL compound solution to the next column successively (8-point dilution series), with mixing. We named these three plates ‘10x lethal plate’. Assay plates were prepared by seeding 4,000 BJ-TERT/LT/ST/RAS^{V12} cells or 3,000 HT1080 cells per well in 32 μL of growth media in black, clear bottom 384-well plates. Cells in assay plates

were first treated with bioactive compounds by transferring 4 µL from the 10x bioactive plate. Then, cells were treated with each lethal compound in a 2-fold dilution series by transferring 4 µL solution from each 10x lethal plate. Assay plates were returned to the culture incubator and maintained for 24 hr before adding alamar blue. Percent growth inhibition (% GI) was calculated from the following formula using fluorescence intensity values.

$$\% \text{ GI} = 100 * (1 - (X - N) / (P - N))$$

X, cells treated with biologically active compound and lethal compound; N, no cells - biologically active compound in growth media; P, no lethal compound - cells were treated with biologically active compound.

shRNA Knock Down Experiments

Virus Production

We used lentiviral plasmids encoding shRNAs targeting VDAC2 (cat.#SHGLY-NM_003375), VDAC3 (cat.#SHGLY-NM_005662), HRAS (cat.#SHGLY-NM_005343), NRAS (cat.#SHGLY-NM_002524), KRAS (cat.#SHGLY-NM_004985 and SHGLY-NM_033360), or TfR1 (Sigma, cat.#SHGLY-NM_003234). All shRNA clones were purchased from Sigma's MISSION® shRNA collection. Plasmid DNA was purified using a HiSpeed Plasmid Midi kit (Qiagen, cat.#12643). On day 1, 2 x 10^6 293T cells were seeded in 10 cm tissue culture dishes; on day 2, 2.8 µg of shRNA-plasmid construct and 2.5 µg of pDelta8.9 and 0.28 µg of pVSV-G helper plasmids were co-transfected into the 293T cells using FuGENE® 6 Transfection Reagent (Roche, cat.#11-814-443-001); on day 3, the medium was replaced with 7.5 mL of VCM (Viral Collection Media) that consists of DMEM supplemented with penicillin and streptomycin (pen/strep), and 30% Hyclone iFCS (Hyclone, cat.#83007-198); on day 4, in the morning, the supernatant containing virus was harvested to empty 50 mL conical tubes and 7.5 mL of fresh VCM was added back to virus producing 293T cell monolayer. We harvested and replaced the VCM again in the evening time; on day 5, in the morning time, we harvested the supernatant and bleached the 293T cell culture. The collected virus supernatant was filtered through a 0.45-µm syringe filter (Nalgene, cat.#190-9945), aliquoted in 2 mL to the cryovials, and stored at -80°C freezer until the time of use.

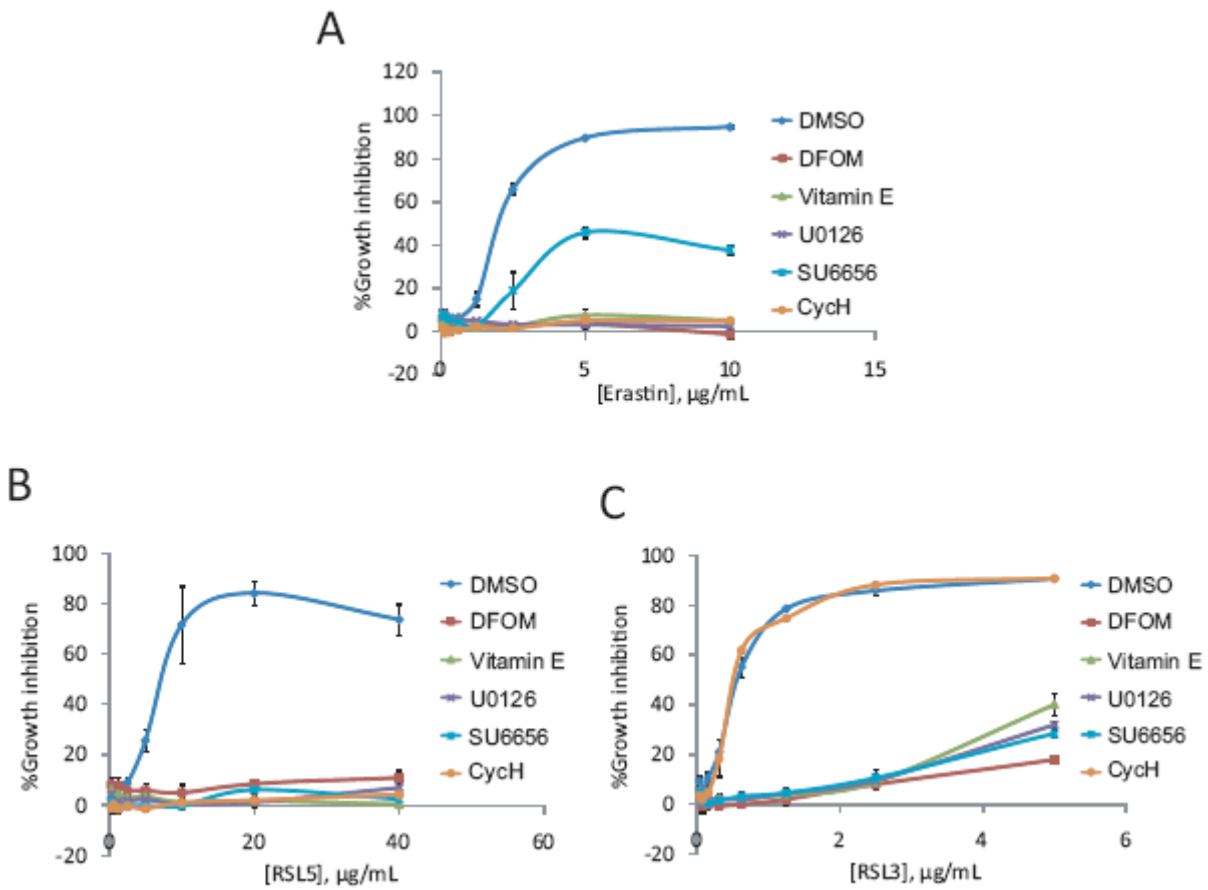
Lentiviral Infection

200,000 HT1080 or Calu-1 cells were seeded on 10 cm tissue culture dishes and the culture was incubated in 37°C, CO_2 incubator for 24 hours. The next day, frozen stocks of virus solution were thawed at 37°C for a couple of minutes and polybrene (Sigma, cat.#H9268) was added at a final concentration of 8 µg/mL. Culture media was replaced with virus/polybrene mix and the culture dish was incubated for two hours with rocking every 30 min. After 2 hours, 10mL of growth media was added to culture dish and the culture was incubated further for 2 days before treatment of compounds.

Table S1. List of Bioactive Molecules

Name	Function	Final Concetration used	Vendor	Cat.#
GSH (Reduced glutathione)	inactivate cisplatin, antioxidant	5mM	Alexis	ALX-157-002-G001
Cyclohexamide	translation blocker	1.5uM	Sigma	C7698
z-VAD(OMe)-fmk	pan-caspase inhibitor	50uM	VWR	100005-274
Boc-D(OMe)-fmk	pan-caspase inhibitor	50uM	MP biomedicals	03FK01101
alpha-tocopherol (Vit. E)	anti-oxidant	100uM	Sigma	T3251
Desferrioxamine Mesylate	Fe mobilizer (inhibits fenton chemistry)	150uM	Sigma	D9533-1G
U0126	MEK1/2 inhibitor	10uM	Alexis	ALX-270-237-M001
SB-203580	SAPK inhibitor	10uM	Alexis	ALX-270-179-M001
Wortmannin	PI3K inhibitor	1uM	Alexis	ALX-350-020-M001
SU6656	Src, Fyn, Lck inhibitor	50uM	Sigma	S9692-5MG
Rapamycin	mTOR inhibitor	0.1uM	Sigma	R0395

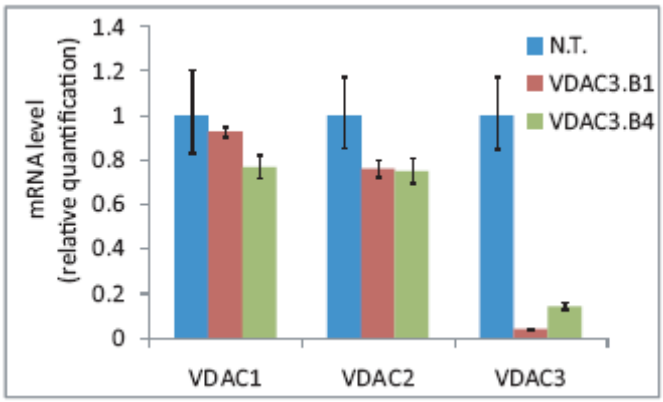
Figure S1. Erastin, RSL5, and RSL3 Share Common Properties in Inducing Oncogenic-RAS-Signal Dependent Lethality in a Natural Tumor Derived Cell Line



Methods

The human fibrosarcoma cell line, HT1080, was treated with erastin (a), RSL5 (b) or RSL3 (c) in the presence of the indicated bioactive compounds. The concentration of each bioactive molecule is listed in Table S1. Percent growth inhibition, measured using alamar blue is shown. Error bars indicate one standard deviation of triplicate data.

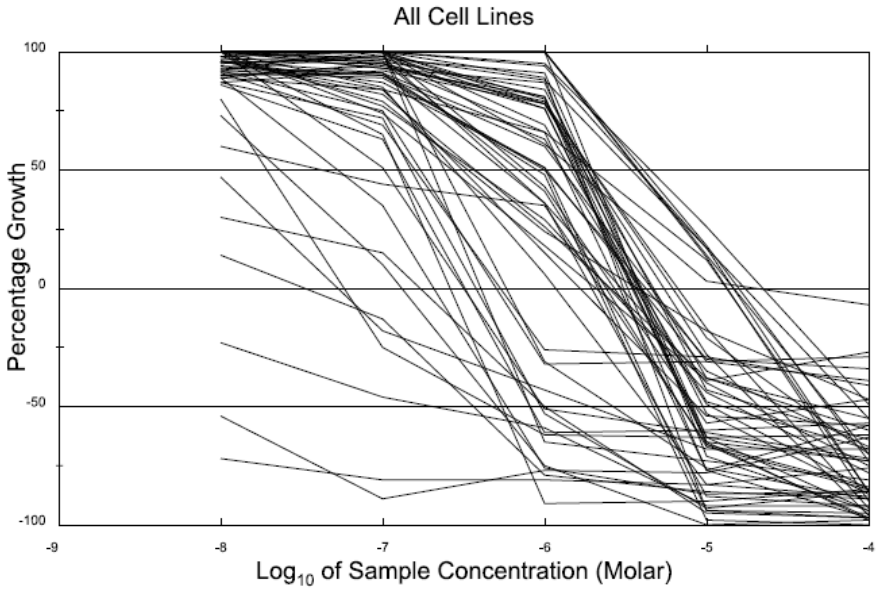
Figure S2. Quantitative-PCR Analysis Validates Specificity of shRNAs Targeting VDAC3



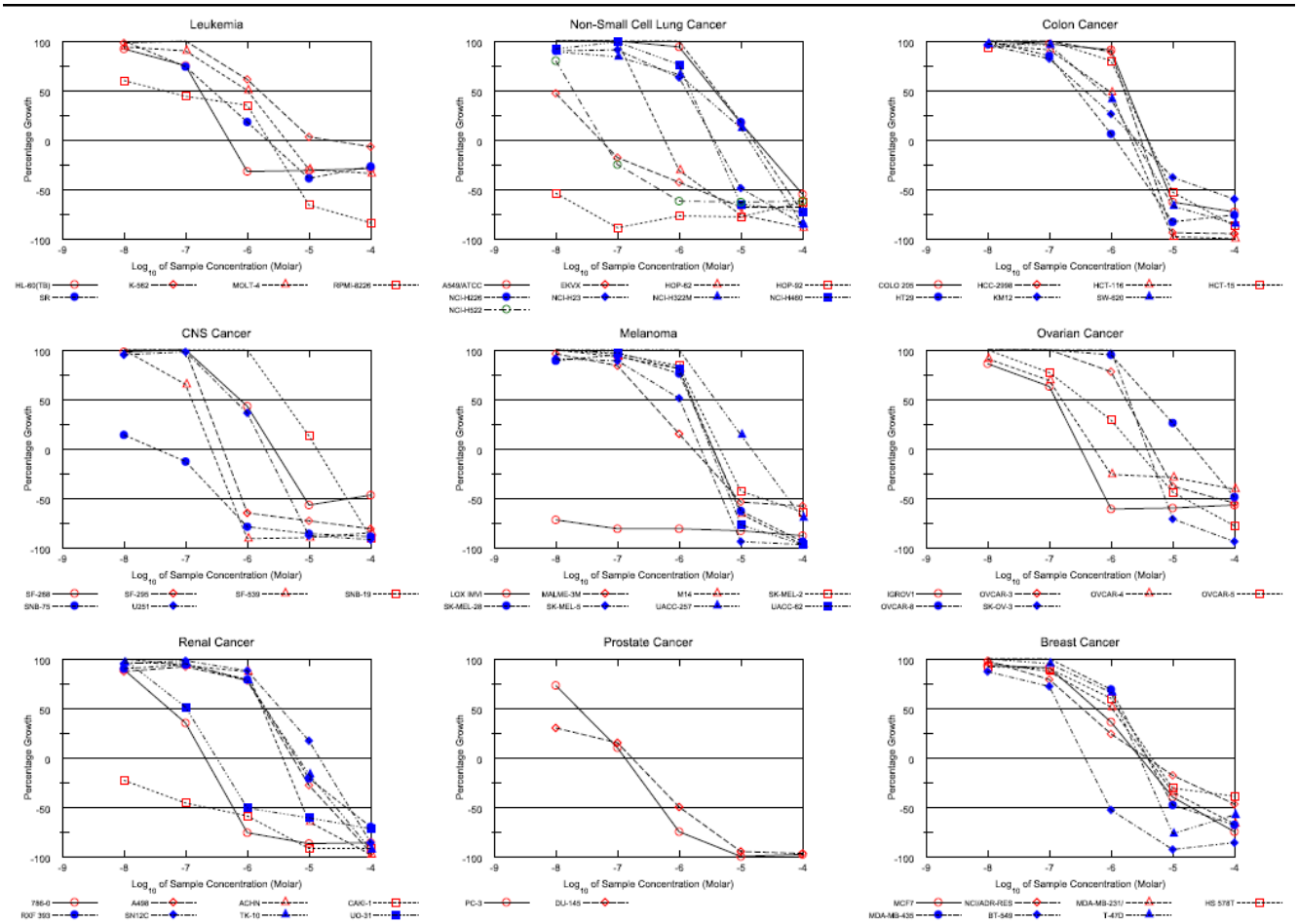
Two independent shRNA clones targeting VDAC3 suppressed erastin and RSL5 induced cell death. As mammalian cells have three VDAC isoforms, VDAC1, VDAC2 and VDAC3, we tested the specificity of shRNAs targeting VDAC3 using Q-PCR method. The figure shows that mRNA level of VDAC3 was significantly reduced upon shRNAs expression, while those of VDAC1 or VDAC2 were not affected by the shRNAs. Error bars indicate maximum and minimum level of relative mRNA amounts.

Figure S3. RSL3 Testing in NCI60 Cell Lines

A. Growth inhibition of 60 different cancer cell lines by RSL3 treatment.



B. Separation of growth inhibition curve based on tissue origin of cancer cell lines



C. Analysis of growth inhibition pattern across the 60 cell lines. Center line represents mean value of all data. Bar graphs pointing rightside indicates that RSL3 is more potent in those cell lines relative to mean value and *vice versa*.

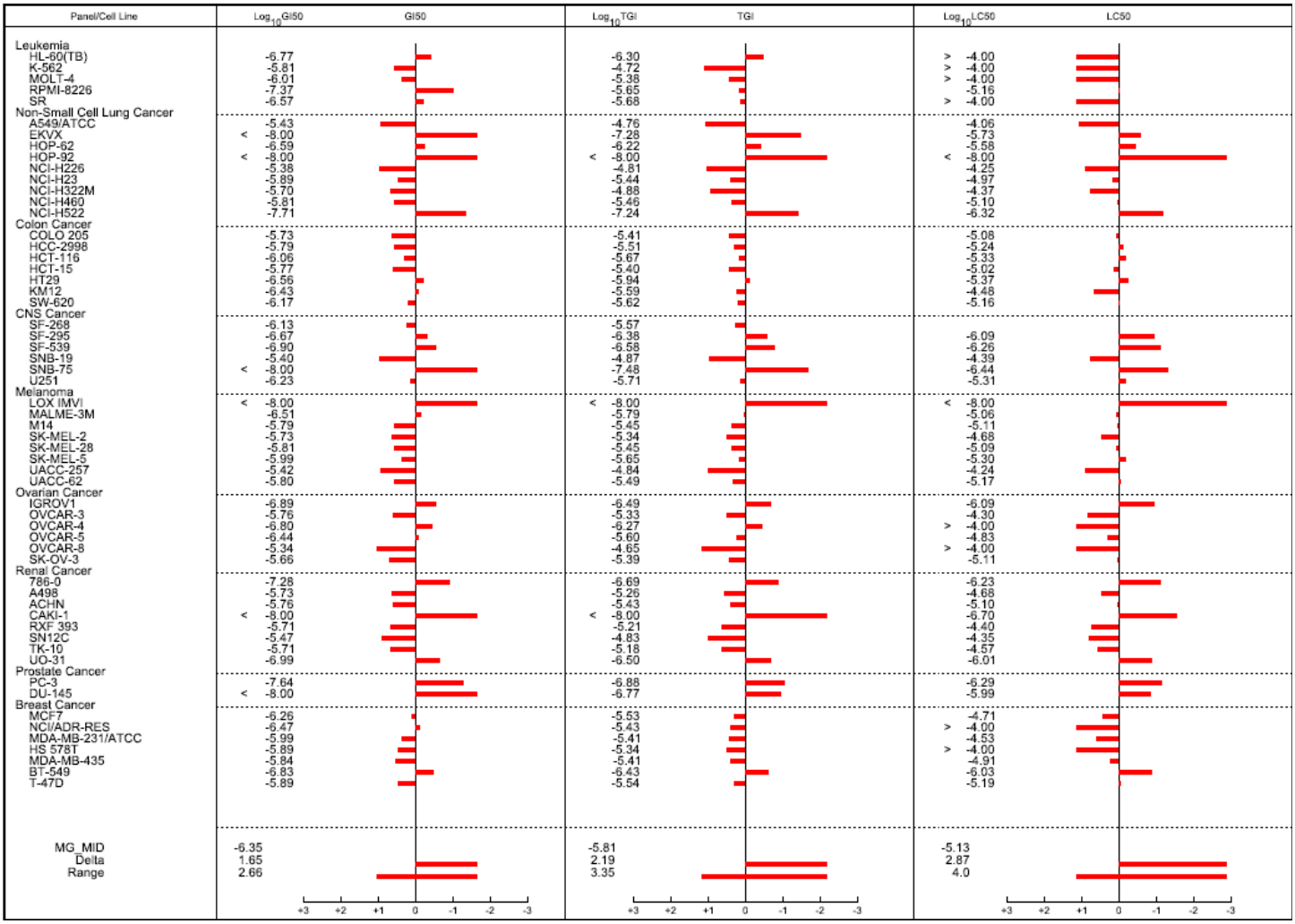
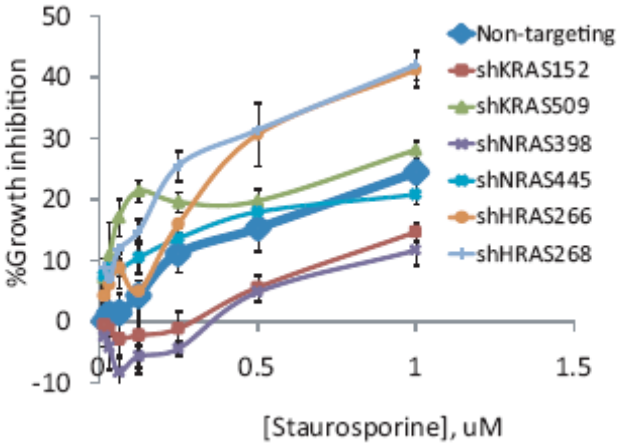


Figure S4. Expression of shRNAs Targeting RAS Did Not Confer Resistance to Staurosporine Treatment

Expression of shRNAs targeting RAS (regardless of isotypes) made Calu-1 cells resistant to RSL3-induced cell death (Figure 6C). To test whether expression of these shRNA clones change cell's physiology such that they become generally resistant to drug treatment, we treated staurosporine to the same cell lines and examined changes in drug sensitivity. As shown in this graph, we could not see the similar level of survival effect in staurosporine treated samples as that in RSL3 treated samples. The percent growth inhibition was determined as described in Fig. 6C. Error bars indicate one standard deviation of triplicate data.



Sequence of Primers Used in Real-Time Quantitative PCR Experiments

VDAC1 F: 5’-CCTGGACAGCAGGAAACAGTAAC-3’
VDAC1 R: 5’-AGGCGTCAGGGTCAATCTGA-3’
VDAC2 F: 5’-TGATTTTGCTGGACCTGCAA-3’
VDAC2 R: 5’-CAGCAAGCCAGCCCTCAT-3’
VDAC3 F: 5’-AATTTCGCCCTGGGTTACAA-3’
VDAC3 R: 5’-TCAGTGCCATCGTTCACATGT-3’
TfR1 F: 5’-GAAAACAGACAGATTTGTCATG-3’
TfR1 R: 5’-CTCTTTTGGAGATACGTAGGG-3’
DMT1 F: 5’-CATCACTATTATGGCCCTCAC-3’
DMT1 R: 5’-GAACATGCCCTTGAGTACCTG-3’
HCP1 F: 5’-GGTCTTTGCCTTTGCCACTATC-3’
HCP1 R: 5’-CAGGTGTGATGACTAATGACAGG-3’
FTH1 F: 5’-CAGATCAACCTGGAGCTCTAC-3’
FTH1 R: 5’-CTTCAAAGCCACATCATCGC-3’
FTL F: 5’-GGCCCTGGAGAAAAAGC-3’
FTL R: 5’-GAAGTGAGTCTCCAGGAAG-3’

The mRNA level of human acidic ribosomal phosphoprotein P0 was measured using the following primers and used as a reference for quantification.
RPLP0 F:5’-ACGGGTACAAACGAGTCCTG-3’
RPLP0 R:5’-GCCTTGACCTTTTCAGCAAG-3’